MECHANISM-BASED INACTIVATION OF HUMAN RECOMBINANT P450 2C9 BY THE NONSTEROIDAL ANTI-INFLAMMATORY DRUG SUPROFEN

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ABSTRACT:
The nonsteroidal anti-inflammatory agent (±)-suprofen [α-methyl-4-(2-thienylcarbonyl)benzeneacetic acid] was evaluated as a P450 2C9 inactivator. (±)-Suprofen inactivated the diclofenac-4-hydroxylation activity of baculovirus-expressed P450 2C9 in a time- and concentration-dependent manner, which was consistent with mechanism-based inactivation. The loss of activity followed pseudo-first-order kinetics and was suprofen- and NADPH-dependent. The kinetic parameters for inactivation \( k_{\text{inact}} \) and \( K_\text{i} \) were 0.091 min\(^{-1}\) and 3.7 \( \mu \)M, respectively, and the partition ratio was 101. Although P450 2C9 substrate S-warfarin partially protected against inactivation, reactive oxygen scavengers such as superoxide dismutase and catalase did not prevent inactivation. Extensive dialysis did not regenerate enzyme activity, suggesting that inactivation proceeded via covalent modification. Inactivated P450 2C9 lost <10% of its ability to form a CO-reduced complex, suggesting that inactivation may have resulted from covalent modification of apoprotein. Addition of exogenous nucleophiles such as glutathione and semicarbazide partially protected against inactivation. Apart from the metabolism of suprofen to 5-hydroxy-suprofen, the formation of a suprofen-glutathione conjugate was also discernible in micosomal mixtures containing glutathione. Time of flight mass spectrometry revealed a protonated monoisotopic mass of 566.1304 for this conjugate, consistent with an elemental composition of \( C_{42}H_{42}N_{10}O_{9}S_2 \). The mass spectrum indicated that conjugation had occurred on the intact thiophene ring, presumably via a thioether linkage. Further evidence for the formation of an electrophilic intermediate in suprofen-P450 2C9 incubations was obtained via the characterization of a novel pyridazine adduct upon addition of semicarbazide to the micosomal mixtures. The pyridazine derivative had a protonated monoisotopic mass of 257.0895 that was consistent with an elemental composition of \( C_{12}H_{16}O_{13}N_{2} \). The formation of the stable pyridazine adduct suggested the generation of an electrophilic \( \gamma \)-thioketo-\( \alpha,\beta \)-unsaturated aldehyde, analogous to that observed during the cytochrome P450-mediated bioactivation of furan. This electrophilic \( \alpha,\beta \)-unsaturated aldehyde represents a possible reactive intermediate that bioalkylates P450 2C9.

Fig. 1. Structural similarities in the NSAID (±)-suprofen and the diuretic tienilic acid.

CO\(_2\)H moiety is essential for P450 2C9 interactions.

(±)-Suprofen [α-methyl-4-(2-thienylcarbonyl)benzeneacetic acid] (Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID\(^1\)) from the 2-arylpropionic acid class of compounds that includes ibuprofen and naproxen. Suprofen was introduced in the market as possessing analgesic, anti-inflammatory, and antipyretic properties comparable to existing NSAIDs but with minimal gastrointestinal liabilities (Todd and Heel, 1985). Within a few months of its release into the marketplace, adverse drug reactions related to acute renal failure were observed (Abreo and LaBarre, 1986; Snyder and Teehan, 1987). The condition manifested itself by the sudden onset of a bilateral acute flank pain syndrome associated with suprofen typically occurring within a few hours of administration and was followed by an acute but reversible renal failure (Hart et al., 1987). Although renal toxicity is a common liability associated with many NSAIDs (Eras and Perazella, 2001), most complications occur as a consequence of chronic administration for extended periods of time and with relatively large doses, often in combination with other analgesics. The acute flank pain syndrome associated with suprofen typically occurred within a few hours of administration and was unlike the nephrotoxic effects of NSAIDs (Hart et al., 1987). Interestingly, the same syndrome has also been reported in patients following administration of the uricosuric diuretic agent, ticrynafen [tienilic acid; 2,3-dichloro-4-(2-thienylcarbonyl)phenoxycetic acid] (see Fig. 1) (Paddack et al., 1980; Powers et al., 1981). Within 1 year of its use in the United

\(^{1}\) Abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; EET, epoxyeicosatrienoic acid; LC/MS-MS, liquid chromatography/tandem mass spectrometry; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; tienilic acid, anti-inflammatory, and antipyretic properties comparable to existing NSAIDs but with minimal gastrointestinal liabilities (Todd and Heel, 1985).

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States, ticrynafen was removed from the market due to its life-threatening hepato- and renal toxicity. Analysis of sera obtained from patients suffering from liver damage following ticrynafen acid exposure revealed the presence of highly specific anti-liver and -kidney microsomal (anti-LKM2) autoantibodies known to specifically recognize P450 2C9 (Lecoeur et al., 1994). Subsequent studies revealed that ticrynafen undergoes a selective P450 2C9-mediated bioactivation reaction on its thiophene ring, resulting in the formation of a reactive intermediate that covalently modifies the enzyme (Jean et al., 1996; Koenigs et al., 1999). Overall, these observations suggest that some of the toxicological consequences following ticrynafen acid administration are mediated through the suicide inactivation of P450 2C9.

Recent studies have confirmed the role of P450 enzymes in the regulation of metabolic pathways responsible for organ function and general homeostasis (Capdevilla et al., 2002). For instance, in the kidney, the P450 2B, 2C, and 2J families are believed to play a pivotal role in the metabolism of prostaglandins to vasoactive epoxide metabolites such as the regioisomeric epoxycycloartenoic acids (EETs) that act locally in the kidney and possess both vasodilatory and vasoconstrictive activity (Capdevilla et al., 2002). Therefore, the hypothesis that suprofen- or ticrynafen-acid-mediated nephrotoxicity might result in part from the suicide inactivation of P450 2C9 or a related 2C isoform in the kidney appears reasonable. Although previous studies have reported on the P450 2C9 substrate and competitive inhibitory properties of many NSAIDs including suprofen (Jones et al., 1996), no details exist on suprofen’s ability to behave as a P450 inactivator. In this paper, we report on the mechanism-based inactivation of P450 2C9 by suprofen and characterization of potential reactive intermediate(s) derived from the bioactivation of its thiophene ring.

Materials and Methods

Materials. (±)-Suprofen, ketoconazole, reduced glutathione, catalase, suprofen diacetate, isoxicamic acid dehydrogenase, and NADP<sup>+</sup> were obtained from Sigma-Aldrich (St. Louis, MO). Human recombinant P450 2C9, semicarbazide hydrochloride, 2-(4-methoxybenzoyl)thiophene, and DL-isocitric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Diclofenac sodium, 4-hydroxydiclofenac, and (S)-warfarin were purchased from BD Gentest (Woburn, MA). High-performance liquid chromatography solvents were of the highest grade commercially available and were used as received. All other chemicals were analytical grade.

Microsomal Incubations. For determinations of kinetic constants, incubations were conducted in a 96-well format utilizing 1.2 ml polyethylene March tubes in a heating block (Boekel Scientific, Feasterville, PA). In general, incubation mixtures (0.4–0.5 ml) contained P450 2C9 (0.4 μM) and a NADPH regenerating system consisting of 10 mM MgCl₂, 0.44 mM NADP<sup>+</sup>, 5 mM DL-isocitric acid, and 0.5 U/ml isoxicamic acid dehydrogenase in 100 mM potassium phosphate buffer (pH 7.4). Several concentrations of suprofen (0–100 μM) were prepared and added in a 50:50 solution of ethanol/H₂O (1% vol/vol) to a secondary incubation mixture (380 μl) containing 2-(4-methoxybenzoyl)thiophene (0.5 μg/ml) as internal standard. After centrifugation (4,000g at 10 min), the supernatant was analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS). Unless stated otherwise, data points represent an average of duplicate determinations.

Enzyme Inactivation Assays. Human recombinant P450 2C9 activity was determined using diclofenac as a substrate. Each primary reaction mixture contained P450 2C9 (0.4 μM) in 100 mM potassium phosphate buffer (pH 7.4), suprofen (0–50 μM), and a NADPH regenerating system. After initiation of catalysis with the NADPH regenerating system (0–15 min), aliquots (20 μl) were periodically transferred to secondary incubation mixtures (380 μl) containing 20 μM diclofenac and the NADPH regenerating system in 100 mM potassium phosphate buffer (pH 7.4). These samples were incubated for 10 min, and the reactions were quenched with acetonitrile containing 2-(4-methoxybenzoyl)thiophene (0.5 μg/ml). 4-Hydroxydiclofenac formation was determined by LC/MS-MS to establish residual enzymatic activity. The 20-fold dilution of the primary incubation mixture allowed differentiation of covalent inactivation from competitive inhibition by suprofen. The calculation of kinetic constants, which describe mechanism-based enzyme inactivators, was performed as previously described (Kitz and Wilson, 1962). Briefly, the relative inhibition of 4-hydroxydiclofenac formation was determined by comparing peak height ratios of time-matched samples with suprofen versus control samples without suprofen. These logarithm of relative inhibition values were plotted versus preincubation time for each concentration of suprofen used, and the slopes were determined by linear regression (SigmaPlot software; SPSS Inc., Chicago, IL). These slopes represent the observed inactivation rate constants, which were used to calculate the half-life of the inactivation reaction. A Kitz-Wilson plot was then constructed using the calculated half-life values (y-axis) and the reciprocal of the associated suprofen concentration (x-axis). The apparent K<sub>x</sub> and k<sub>inact</sub> values were determined from the reciprocal of the y-axis intercept and the negative reciprocal of the x-axis intercept of the Kitz-Wilson plot, respectively.

LC/MS-MS Assay for 4-Hydroxydiclofenac Analysis. 4-Hydroxydiclofenac formation was assessed by electrospray tandem quadrupole mass spectrometry in line with a LC system. The LC system consisted of two Hewlett Packard 1100 Series binary channel pumps (Hewlett Packard, Palo Alto, CA) used in single-channel operation to provide solvent delivery of 0.1% acetic acid in H₂O/acetonitrile (95:5) (pump 1) and 0.1% acetic acid in H₂O/acetonitrile (5:95) (pump 2). A Gilson 215 autosampler (Gilson Medical Electronics, Middleton, WI) injected 20 μl of sample onto the LC system, and analytes were separated using a Phenomenex Hypersil C18, 30 × 2 mm, 5-μm column (Phenomenex, Torrance, CA). The LC was programmed such that 100% of solvent from pump 1 ran for the first 30 s to load analytes and flush out solvent front material postcolumn to waste. At 30 s, solvent delivery was switched to 100% pump 2, which subsequently eluted analytes from the column. Postcolumn effluent was switched to the mass spectrometer for analysis. At 1 min, solvent delivery was switched back to pump 1 and equilibrated for 30 s before injection of the next sample. Flow of pump 1 was 1 ml/min and flow of pump 2 was 1.5 ml/min. Under these conditions, 4-hydroxydiclofenac and 2-(4-methoxybenzoyl)thiophene (internal standard) eluted at 0.75 and 0.85 min, respectively. Total run time was 1.5 min. Detection of analytes was achieved using a Sciex model 3000 liquid chromatography/tandem mass spectrometer (LC/MS/MS) (MDS Sciex, Concord, ON, Canada). Effluent was split before introduction into the turbo-ion spray source so that flow rate into the mass spectrometer was maintained such that 100% of solvent from pump 1 was used for the next analysis. Ionization was conducted in the positive ion mode at a source temperature of 200°C. The ion spray voltage was set at 4.0 kV, the orifice voltage was optimized at 45 eV, and the collision energy was adjusted to ~30 eV. 4-Hydroxydiclofenac and the internal standard 2-(4-methoxybenzoyl)thiophene were analyzed using multiplex reaction monitoring at mass ranges m/z 312→231 and m/z 219→135, respectively. The assay had a linear dynamic range of 0.05 to 5.0 μM.

Addition of Trapping Agents and Metabolite Identification. Protection against inactivation of P450 2C9 by suprofen via the addition of exogenous trapping agents including glutathione (5 mM) and semicarbazide (1 mM), as well as the effect of NADPH, suprofen diacetate (700 μM), and catalase (2000 units) on inactivation was assessed by conducting incubations (0.5 ml) with suprofen (50 μM) for 30 min and transferring 20 μl to a secondary incubation (as described above) to estimate residual activity. For metabolite inactivation, the incubation volume was increased to 1 ml, suprofen and P450 2C9 concentrations were adjusted to 100 μM and 0.5 μM, respectively, and the length of the incubation was increased to 45 min. The reactions were quenched with 2 ml of acetonitrile, centrifuged for 10 min at 4,000g. After evaporation of the supernatant under nitrogen gas, the samples were reconstituted (100 μl) in mobile phase (95:5; 10 mM ammonium formate containing 0.1% formic acid/acetonitrile) before analysis by LC/MS-MS.

Spectrophotometric Analysis. Primary reaction mixtures containing P450 2C9 (0.4 μM) and suprofen (50 μM) in the presence or absence of NADPH or other trapping agents were incubated for 30 min (to allow for maximal inactivation), after which time, the samples were placed on ice.
were treated with CHAPS (1% final) and reduced with dithionite before obtaining a reference spectrum on a Shimadzu UV-visible spectrophotometer. Samples were then purged with CO gas for 30 s and a second spectrum was obtained to determine the spectral difference. Absolute spectral values were determined by scanning from 400 to 500 nm relative to catalase, CHAPS, superoxide dismutase, glutathione, semicarbazide, and phosphate buffer (pH 7.4) in the reference.

Substrate Protection Against Inactivation. Incubations of (z)-suprofen (25 μM) were conducted in the presence of S-warfarin, a P450 2C9 substrate, at molar ratios of 1:0, 1:1, 1:2, and 1:4 (suprofen/S-warfarin) in the primary incubation. Aliquots (20 μl) were periodically removed and assayed for residual activity in a secondary incubation as described above.

Partition Ratio. Incubations were conducted at various concentrations of suprofen (0–200 μM) for 30 min to ensure complete inactivation. Aliquots (20 μl) were periodically removed and assayed for residual activity in a secondary incubation as described above. The percentage activity remaining was then plotted versus the ratio [suprofen]/[P450 2C9]. As previously described (Kent et al., 1998), extrapolating the intercept of the linear regression of lower suprofen concentrations and the horizontal line from saturating suprofen concentrations yielded the partition ratio.

Irreversibility of Inactivation. Separate 1-ml incubations (in the presence or absence of an NADPH regenerating system) containing (z)-suprofen (200 μM) were incubated for 30 min and assayed for residual activity as described above. The remaining portion was subjected to overnight dialysis against 25 mM potassium phosphate buffer (pH 7.4) at 5°C using a Slide-A-Lyzer dialysis cassette (Pierce Chemical, Rockford, IL) and then reassessed for activity the next day. Activity was assessed relative to the minus NADPH control.

18O Incorporation Studies. The source of oxygen for the formation of the major 5-hydroxy metabolite of (z)-suprofen was characterized using H18O and 18O2 gas (Isotec Inc., Miamisburg, OH). For H18O studies, 0.2-ml incubations containing 100 μM (z)-suprofen were performed in a buffer consisting of 50% (v/v) 200 mM potassium phosphate (pH 7.4)/H18O. After 45 min, the reactions were quenched with 0.4 ml of acetonitrile, and the samples were analyzed for metabolites using the method described above. Studies utilizing 18O2 gas as the source of oxygen were conducted in a sealed 10-ml round-bottomed flask. Incubations [3 ml, 100 μM (z)-suprofen] were flushed and purged with nitrogen/vacuum five times before addition of 18O2 gas. Internal pressure was monitored during the flushing cycles and during the incubation with a syringe barrel equipped with a balloon. The reaction was initiated with the addition of a NADPH regenerating system and stopped at 45 min with acetonitrile (6 ml), both of which were added via a syringe. Metabolite identification was conducted as outlined above.

Metabolite Identification by LC-MS/Quadropole Time of Flight Mass Spectrometry (LC-MS/TOF). Metabolite identification and the characterization of trapped reaction products were assessed by electrospray hybrid quadrupole time of flight mass spectrometry. Analytes were separated using a Hewlett Packard 1100 Series quad channel LC pump utilizing a binary gradient of 10 mM ammonium formate containing 0.1% formic acid/CH3CN (95:5) (solvent A) and 10 mM ammonium formate containing 0.1% formic acid/CH3CN (5:95) (Solvent B). The gradient was programmed as follows: (solvent A/solvent B) 100:0 for the first 3 min; ramped to 10:90 in 15 min; held at 10:90 for 5 min; then ramped to 100:0 over the next 7 min. A Zorbax RX-C18 column, 4.6 mm × 50 mm, 5 μm, was used for the separation. The LC-delivered solvent at a rate of 1 ml/min and a 50-μl injection volume was provided by a CTC-PAL autosampler (CTC Analytics, Zwingen Switzerland). Postcolumn effluent was directed to a photodiode array detector (Hewlett Packard 1010 Series) set to acquire at 252 to 254 nm. Flow from the photodiode array was then split to a Micromass qTOF hybrid quadrupole time of flight mass spectrometer (Micromass Inc., Beverly, MA) equipped with an electrospray source. Two splitters were placed in line to allow the infusion (2–5 μl/min) of a lock mass solution (10 μM diclofenac sodium, 10 μM ketocozonel in 50:50 10 mM ammonium formate containing 0.1% formic acid/CH3CN) and to split flow such that the effluent to the mass spectrometer was introduced at a rate of 50 μl/min. This setup provided postcolumn addition of appropriate concentrations of lock masses prior to introduction into the electrospray source. The capillary voltage was set at 2.5 kV and the cone voltage was optimized to 35 eV. The collision gas (argon) was left on for all TOF acquisitions total ion (MS) and collision-induced dissociation (CID) (MS-MS) experiments. Collision-induced dissociation spectra were obtained using a collision energy of 25 eV. All other quadrupole and TOF voltages were optimized for standard performance specifications [resolution ~5,000 full width at half maximum, mass accuracy <10 ppm with internal standard (lock mass)]. Acquisition, centroiding, and elemental analysis of spectra were completed using MassLynx v.3.5 (Micromass Inc.).

Results

Mechanism-Based Inactivation of P450 2C9 by (z)-Suprofen. The kinetics for P450 2C9 inactivation was studied by measuring the loss of diclofenac-4-hydroxylase activity. Human recombinant P450 2C9 was inactivated by suprofen in a NADPH-, time-, and concentration-dependent manner (Fig. 2). Since there was a 20-fold dilution in the enzyme concentration from the transfer of an aliquot from the primary to the secondary reaction mixture, the competitive inhibitory effects of suprofen within the secondary reaction mixture were minimal. The inactivation followed pseudo-first-order kinetics. The kinetic constants were determined from a double-reciprocal plot of the inverse of the initial rates of inactivation as a function of the reciprocal of the suprofen concentration (Fig. 2, inset). The concentration that produced half-maximal inactivation (K1/2) was 3.7 μM, and the maximal rate of inactivation at saturation (k 1/2) was 0.091 min−1. Thus, the half-life of inactivation (t 1/2) was 7.6 min. At higher suprofen concentrations (≥100 μM) or longer inactivation times (≥20 min), the reaction kinetics became biphasic (data not shown). Therefore, the initial linear rates were used to calculate the kinetic constants. The diclofenac-4-hydroxylase activity remaining after inactivation of P450 2C9 with 50 μM suprofen for 30 min was compared with the P450 content determined from the reduced CO spectrum (Table 1). No inactivation or loss of CO binding was observed in the control samples that had been incubated without suprofen or NADPH. Inactivation of P450 2C9 by suprofen was NADPH-dependent, and after a 30-min incubation, a 78% decrease in enzymatic activity was discernible without any significant loss in spectrally detectable enzyme.

Partition Ratio. The number of molecules of suprofen metabolized per molecule of 2C9 inactivated, i.e., the partition ratio, was estimated from the percentage of remaining activity following incubation with various concentrations of suprofen. The reactions were allowed to progress until the inactivation was complete (30 min). The turnover number was estimated by plotting the percentage of remaining activity as a function of the molar ratio of suprofen to P450 2C9 (Fig. 3), as previously demonstrated with other mechanism-based inactivators (Kent et al., 1998; Regal et al., 2000). In contrast to the classic turnover number of enzyme kinetics (k cat), the turnover number in the present analysis represents the number of inactivator molecules required for complete inactivation and is directly related to the partition ratio (turnover number = partition ratio + 1). The partition ratio was estimated to be 101.

Substrate Protection from Inactivation of P450 2C9 by Suprofen. The effect of the alternate P450 2C9 substrate (S)-warfarin on the inactivation of P450 2C9 by suprofen was studied (Fig. 4). Incubation of P450 2C9 with 25 μM suprofen together with increasing molar ratios of (S)-warfarin (1:0, 1:1, 1:2, and 1:4 suprofen/(S)-warfarin) resulted in a decrease in the rate of inactivation of P450 2C9 as determined by measuring the level of diclofenac-4-hydroxylation. Incubation of P450 2C9 for 15 min with suprofen and (S)-warfarin at a molar ratio of 1:4 resulted in a 10% loss in activity, whereas 70% of the P450 2C9 activity was lost in the absence of (S)-warfarin.

Irreversibility of P450 2C9 Inactivation by Suprofen. Incubation of P450 2C9 with 200 μM suprofen and NADPH for 30 min
resulted in a loss of >90% of the diclofenac-4-hydroxylase activity relative to minus NADPH controls. NADPH and unbound suprofen were removed from the samples by extensive dialysis against potassium phosphate buffer (pH 7.4) at 5°C overnight. Less than 10% of diclofenac-4-hydroxylase activity was discernible in the washed, inactivated samples as compared with dialyzed control samples, which were previously incubated without NADPH. This observation suggests that inactivation of P450 2C9 by suprofen is irreversible and most likely involves covalent modification of the enzyme.

**Effect of Exogenous Nucleophiles and Reactive Oxygen Scavengers on the Inactivation of P450 2C9 by Suprofen.** The effect of exogenous nucleophiles on the rate of inactivation of P450 2C9 by suprofen was investigated by coincubating P450 2C9 with 50 μM suprofen and 5 mM glutathione or 1 mM semicarbazide. Both glutathione and semicarbazide did not prevent P450 2C9 inactivation by

**TABLE 1**

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage of Control Activity</th>
<th>Percentage of P450 Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>93</td>
<td>103</td>
</tr>
<tr>
<td>Suprofen (50 μM)</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>Suprofen (50 μM) + NADPH</td>
<td>22</td>
<td>99</td>
</tr>
<tr>
<td>Suprofen (50 μM) + NADPH + glutathione (5 mM)</td>
<td>27</td>
<td>96</td>
</tr>
<tr>
<td>Suprofen (50 μM) + NADPH + semicarbazide (1 mM)</td>
<td>33</td>
<td>94</td>
</tr>
<tr>
<td>Suprofen (50 μM) + NADPH + SOD (700 units)</td>
<td>24</td>
<td>not determined</td>
</tr>
<tr>
<td>Suprofen (50 μM) + NADPH + catalase (2,000 units)</td>
<td>26</td>
<td>not determined</td>
</tr>
</tbody>
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SOD, superoxide dismutase.
suprofen. Thus, the maximal rate of inactivation ($k_{\text{max}}$) and the time required to inactivate half the enzyme molecules ($t_{1/2}$) in the presence of glutathione was 0.065 min$^{-1}$ and 10.7 min, whereas in the presence of semicarbazide, $k_{\text{max}}$ and $t_{1/2}$ were 0.051 min$^{-1}$ and 13.6 min, respectively ($k_{\text{max}}$ and $t_{1/2}$ in the absence of nucleophiles were 0.091 min$^{-1}$ and 7.6 min). Changes in $K_i$ in the presence of the two exogenous nucleophiles were also minimal ($K_i$ without nucleophiles $\sim$3.7 $\mu$M; $K_i$ (+glutathione) $\sim$3.6 $\mu$M; $K_i$ (+semicarbazide) $\sim$3.8 $\mu$M). Under the conditions leading to maximal inactivation, minimal changes in the inactivation were also observed in the presence of reactive oxygen scavengers such as superoxide dismutase and catalase. Furthermore, maximal inactivation did not lead to a substantial loss in the ability of P450 2C9 to form a reduced CO complex (see Table 1).

Identification of Metabolites and Characterization of Reactive Intermediates. LC/MS-MS analysis of the incubation mixture containing 5 mM glutathione by LC-MS/qTOF revealed the formation of a glutathione adduct with an observed protonated monoisotopic mass of 566.1304 (calculated mass = 566.1267; error $\sim$3.7 ppm), which was consistent with an elemental composition of C$_{34}$H$_{37}$N$_3$O$_9$S$_3$. The molecular ion at 566 represented the addition of the molecular mass of glutathione to that of intact suprofen. The CID spectrum of this adduct was similar to the one previously reported for authentic 5-hydroxysuprofen (Mori et al., 1984). Besides phase II glucuronidation of the carboxylic acid group, formation of 5-hydroxysuprofen also constitutes a major mechanism of suprofen clearance in humans (Fig. 5, pathway A or B $\rightarrow$ D) (Mori et al., 1985).

Analysis of the incubation mixtures containing 5 mM glutathione by LC-MS/qTOF revealed the formation of a glutathione adduct with an observed protonated monoisotopic mass of 566.1304 (calculated mass = 566.1267; error $\sim$3.7 ppm), which was consistent with an elemental composition of C$_{34}$H$_{37}$N$_3$O$_9$S$_3$. The molecular ion at 566 represented the addition of the molecular mass of glutathione to that of intact suprofen. The CID spectrum of this adduct (Fig. 6) revealed characteristic fragment ions at $m/z$ = 491 (loss of glycine, 75 Da), $m/z$ = 437 (loss of pyroglutamate, 129 Da), $m/z$ = 293 (cleavage between the cysteinyl C-S bond), and $m/z$ = 260 (intact suprofen), suggesting that glutathione conjugation had occurred on the thiophene ring, presumably via a thioether linkage (such as the regioisomers 4 and 5, Fig. 5, B $\rightarrow$ C).

Analysis of the incubation mixture containing 1 mM semicarbazide by LC-MS/qTOF revealed the formation of a metabolite with an observed protonated monoisotopic mass of 257.0895 (calculated mass = 257.0895; error $\sim$3.1 ppm), which was consistent with an elemental composition of C$_{14}$H$_{13}$O$_3$N$_2$. The CID spectrum of this metabolite (Fig. 7) indicated the presence of an additional acylium ion fragment at $m/z$ = 107 besides the characteristic acylium ion fragments at 177 and 105 in suprofen. The additional fragment was consistent with the pyridazine acylium ion fragment (see Fig. 7). Based on its mass spectral characteristics, we proposed the structure of the P450 2C9/suprofen/semicarbazide reaction product to be $\alpha$-methyl-4-(3-pyridinylcarbonyl)benzene acetic acid (6) (Fig. 5, Pathway B $\rightarrow$ E $\rightarrow$ F).

$^{18}$O Incorporation during the Formation of the 5-Hydroxysuprofen Metabolite. The source of oxygen required for the formation of 5-hydroxysuprofen ([M + H]$^+$ = 277) metabolite was characterized using H$_2$$^{18}$O and $^{18}$O$_2$ gas. Incubation mixtures in H$_2$$^{18}$O did not reveal any incorporation of $^{18}$O. In contrast, LC/MS-MS of the incubation mixture in the presence of $^{18}$O$_2$ gas revealed that 96.8% of the 5-hydroxysuprofen metabolite formed displayed an intense (M + H)$^+$ at 279, characteristic of the incorporation of one $^{18}$O atom.

Discussion

Based on the observations on the covalent binding of suprofen acyl glucuronide to albumin and rat renal tissue, it was proposed that this major metabolite in humans might be responsible for the unique nephrotoxicological consequences associated with this NSAID (Smith and Liu, 1993, 1995). This proposal, however, contradicts the well-established notion that adverse drug reactions from protein modification by acyl glucuronides are immune-mediated (Ju and Uetrecht, 2002), whereas the acute flank pain syndrome observed does not appear to be derived from an immune response. Furthermore, acyl

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**Figure 3.** Loss of P450 2C9 activity as a function of the ratio of (±)-suprofen to enzyme.

P450 2C9 was incubated with varying (±)-suprofen concentrations for 30 min until the inactivation was essentially complete. Extrapolating the intercept of the linear regression of lower suprofen concentrations and the horizontal line from saturating suprofen concentrations yielded the partition ratio.

**Figure 4.** Substrate protection against P450 2C9 inactivation by (±)-suprofen.

The reaction mixtures were as described under Materials and Methods. Portions of the samples were removed from the primary reaction mixture at the time points indicated and assayed for catalytic activity remaining. The molar ratios of substrates were: no (S)-warfarin or (±)-suprofen ( ), (S)-warfarin/(±)-suprofen = 0:1 ( ), (S)-warfarin/(±)-suprofen = 1:1 ( ), (S)-warfarin/(±)-suprofen = 2:1 ( ), and (S)-warfarin/(±)-suprofen = 4:1 ( ).
glucuronidation constitutes a major portion of metabolic clearance of all carboxylic acid-containing NSAIDs including naproxen, ibuprofen, ketoprofen, and diclofenac, and several of them have been shown to bind albumin and other biomacromolecules, yet these drugs do not share suprofen-like nephrotoxicity. Furthermore, recent studies have established an in vitro assessment of reactivity of various acyl glucuronides in which an excellent correlation has been established between the amount of covalent binding with the aglycone formation rate when weighted by the percentage of isomerization. (Bolze et al., 2002). The acyl glucuronide derived from tolmetin was ranked as the most reactive, whereas the acyl glucuronide obtained from furosemide was determined to be the least reactive. The reactivity of suprofen acyl glucuronide was fairly low and comparable to that of the acyl glucuronides derived from the well tolerated NSAIDs ibuprofen and ketoprofen.

Finally, recent studies (Mansuy et al., 1984) have indicated that 5-hydroxytienilic acid, the major urinary metabolite of tienilic acid, and not tienilic acid acyl glucuronide, is responsible for the adverse drug reactions associated with that agent. Consequently, these observations and the obvious structural similarity between suprofen and tienilic acid led us to evaluate the alternate possibility that suprofen could function as a suicide inactivator of P450 2C9 via bioactivation of its thiophene ring. This hypothesis seemed attractive since renal impairment following the suicide inactivation of P450 2C9 has been demonstrated. For example, suicide inactivation of the P450-catalyzed \( \omega \)-hydroxylation of arachidonic acid in rat renal tissue by 17-octadecynoic acid markedly reduces the formation of vasoactive 20-hydroxyicosatrienoic acid and EETs (Zou et al., 1994b). In addition, in vivo infusion of 17-octadecynoic acid into the renal artery of rats also increases urinary and sodium excretion and papillary blood flow (Zou et al., 1994a).

In humans, it is clear that P450 2C enzymes expressed in high concentrations in the kidney play a major role in the regioselective epoxidation of the polyunsaturated fatty acid substrate arachidonic acid to the corresponding 14,15-, 11,12-, and 8,9-EETs (Daikh et al., 1994; Rifkind et al., 1995). EETs demonstrate a broad and contrasting spectrum of biological activities, including both vasodilatory and vasoconstrictive influences. This, combined with vasoactive arachidonic acid metabolites biosynthesized via the COX pathway, provides a complex balance of endogenous messengers involved in the regulation of renal tubular and vascular function. Thus, it is tempting to speculate that the combination of COX-1/-2 inhibition and the suicide inactivation of P450 2C isozymes in the kidney by suprofen may result in predisposing physiological factors that may collectively result in the nephrotoxic effects. However, the possibility that reactive intermediates derived from the P450 2C9-catalyzed suprofen bioactivation are modifying renal biomacromolecules other than P450 and COX-1/-2 cannot be ruled out. Overall, the inhibitory effect of suprofen on other renal P450 enzymes (e.g., 2B, 4A, and 2J) important in polyunsaturated fatty acid metabolism remains to be elucidated.
The inactivation of P450 2C9 by suprofen followed pseudo-first-order kinetics, was time- and concentration-dependent, and required NADPH. Addition of catalase and superoxide dismutase did not prevent inactivation, suggesting that reactive oxygen species (hydrogen peroxide and superoxide) were not involved in the inactivation event. Under experimental conditions that led to significant loss of diclofenac-4-hydroxylase activity, the level of CO binding was not reduced. This result indicated that inactivation of P450 2C9 by suprofen occurred mainly due to a modification of the apoprotein rather than the heme prosthetic group. The modification by the reactive suprofen intermediate appeared to be covalent since extensive washing did not lead to a recovery of enzymatic activity. The absolute requirement of NADPH for inactivation and the protection against inactivation offered by warfarin confirmed the need for suprofen bioactivation prior to covalent modification. These results also suggested that the P450 2C9 active site was the place where the inactivating event occurred. The detection of glutathione conjugates and the pyridazine metabolite in suprofen-P450 2C9 incubations is consistent with bioactivation of this NSAID to electrophilic intermediate(s) capable of alkylating the enzyme.

The clinical consequences of mechanism-based inactivation of P450 have received much attention of late. In vitro models have demonstrated that potential drug-drug interactions incurred through mechanism-based inactivation are typically underestimated using standard competitive inhibition models (Mayhew et al., 2000). Plasma concentrations of suprofen have been shown to exceed 50 μM after a single 200-mg dose (Chu, 1983). Although protein binding is estimated at 99%, free concentrations of 0.5 μM are significant for inactivation based upon a $K_I$ of 3.7 μM and a $k_{inact}$ of 0.091 min$^{-1}$.

Our $^{18}$O$_2$ labeling studies as well as those resulting in the characterization of the glutathione conjugates and the pyridazine adduct provide useful mechanistic insight into the nature of the reactive species derived from suprofen bioactivation. The proposed mechanism of P450 2C9 inactivation by the related thiophene derivative tienilic acid involves the P450-catalyzed oxidation on the thiophene sulfur, leading to a highly reactive thiophene-S-oxide intermediate 1 (Fig. 5, pathway A) (Lopez-Garcia et al., 1994; Mansuy, 1997). 1,6-Michael addition of an active site amino acid residue at the C5 position of the reactive thiophene-S-oxide followed by dehydration affords the inactive P450 2C9/tienilic acid adduct 2. Alternately, nucleophilic 1,6-addition of water or reduced glutathione followed by dehydration would afford 5-hydroxytienilic acid (3), the major metabolite of tienilic acid, and the 5-glutathionyltienilic acid adduct 4, respectively.

Electrospray mass spectrometric analysis of tienilic acid/P450 2C9 reaction mixtures, however, reveals the molecular mass of the P450 2C9/tienilic acid adduct to be 16 mass units higher than would be expected from a simple coupling of tienilic acid with P450 2C9 according to Fig. 5, pathway A (Koenigs et al., 1999). This finding suggests that each tienilic acid molecule covalently bound to P450
2C9 contains a hydroxyl group, on the basis of which, an alternate mechanism of inactivation (Fig. 5, pathway B) has been suggested (Koenigs et al., 1999; Dalvie et al., 2002) and partially confirmed in studies involving the chemical oxidation of thiophenes (Treiber et al., 2003). In this proposal, P450 2C9-catalyzed oxidation of the thiophene ring would afford the reactive thiophene-4,5-epoxide, nucleophilic ring, opening of which by an active site residue would produce a P450 2C9 adduct with a molecular mass consistent with the observed mass spectrometric results (Fig. 5, pathway C). Alternatively, addition of glutathione across the epoxide followed by dehydration would lead to the regioisomeric thioethers 4 and/or 5, respectively (see Fig. 5, pathway C). The thiophene epoxidation pathway is attractive since it also accounts for 5-hydroxythienilic acid formation (see Fig. 5, pathway C). The thiophene epoxidation pathway is attractive since it also accounts for 5-hydroxythienilic acid formation that could result via intermediate 6 (Fig. 5, pathway D). Our results on the 18O uptake experiments with suprofen strongly suggest that the source of oxygen in 5-hydroxysuprofen is derived almost exclusively from 18O2 and not from H218O. Although the 18O studies are consistent with the epoxidation pathway, they do not prove the existence of the epoxide intermediate, since direct hydroxylation on the thiophene ring system could also result in the formation of 6. Overall, this finding does contradict the proposed pathway of 5-hydroxythiophene formation via the 1,6-Michael addition of H218O to the thiophene-S-oxide followed by dehydration. Additional support for the thiophene epoxidation pathway stems from the characterization of the novel pyridazine derivative 8 during the P450 2C9-catalyzed bioactivation of suprofen. A proposed mechanism for the formation of the pyridazine adduct involves the spontaneous ring opening of the thiophene-4,5-epoxide intermediate to a reactive γ-thioketo-α,β-unsaturated aldehyde intermediate 7 that upon condensation with semicarbazide will afford 8 (Fig. 5, pathway B→E→F).

Overall, this is the first report on the characterization of a stable adduct derived from a P450-mediated thiophene ring opening reaction. Our observations on the P450-mediated thiophene ring scission in suprofen to the reactive γ-thioketo-α,β-unsaturated aldehyde is a reasonable outcome based on P450-catalyzed bioactivation of analogous furan derivatives that leads to cis-2-butene-1,4-dialdehyde reactive intermediates following ring scission of the initial furan epoxide species (Chen et al., 1997). In the case of furan analogs, the reaction of the corresponding cis-2-butene-1,4-dial metabolites with proteins and DNA is thought to be responsible for the toxicological and carcinogenic outcomes of furan and its derivatives (Byrns et al., 2002). On the basis of this hypothesis, it is reasonable to speculate the involvement of the γ-thioketo-α,β-unsaturated aldehyde as the electrophilic intermediate derived from the bioactivation of suprofen that covalently binds to P450 2C9, in addition to or as an alternative to the reactive thiophene epoxide metabolite. Verification of apo-P450 2C9 adduction by suprofen is currently in progress using electrospray ionization mass spectrometry.

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References


